Effect of *Alternaria solani* Exudates on Resistant and Susceptible Potato Cultivars from Two Different pathogen isolates

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Early blight of potato, caused by *Alternaria solani*, is a ubiquitous disease in many countries around the world. Our previous screening of several Iranian potato cultivars found that significant variation in resistance phenotypes exists between two cultivars: resistant 'Diamond' and susceptible 'Granula'. Our previous analysis of five different pathogen isolates also identified varying degrees of aggressiveness regardless of the host cultivar. Here, a bioassay was used to study the role of liquid culture exudates produced *in vitro* on pathogenicity and elicitation of disease symptomology in seedlings as well as detached leaves. Responses of host genotypes to the exudates of the two *A. solani* isolates were significantly different. Detached leaves of the resistant cultivar 'Diamond' elicited fewer symptoms to each isolate when compared to the susceptible cultivar 'Granula'. Interestingly, the phytotoxicity effect of the culture filtrate from the more aggressive isolate A was higher than from isolate N suggesting an increased concentration or strength of the toxins produced. Our results are significant because they indicate a correlation between symptoms elicited by *A. solani* phytotoxins and their aggressiveness on the host.

Keywords : *Alternaria solani*, bioassay, early blight, pathogenicity, phytotoxicity.

Early blight, caused by *Alternaria solani* is a serious and important plant disease in warm and humid regions (Scherf and MacNab, 1986). The main hosts of *A. solani* are solanaceous crops including tomato, potato, eggplant, and pepper (Ellis and Gibson, 1975; Neergaard, 1945). The disease is characterized by formation of dark brown lesions and necrosis of leaf tissue between the veins. Host colonization is facilitated by enzymes (cellulases and pectin methyl galacturonase) that degrade the host cell wall and by

phytotoxins that facilitate the necrotrophic growth habit of the fungus (Rotem, 1994). Fungi of the genus *Alternaria* produce host-specific and host non-specific toxins involved in virulence or pathogenesis, which affect structural components of the host tissues including the plasma membrane, chloroplasts, and mitochondria (Hanneke et al., 1988; Lynch et al., 1991; Matern et al., 1978; Otani et al., 1995). Eleven toxins have been identified in culture filtrates of *A. solani* (Montemurro and Visconti, 1992). Among these, alternaric acid and solanapyrone A, B, and C are able to induce necrotic symptoms similar to EB symptoms (Montemurro and Visconti, 1992). Alternaric acid is one of the major metabolites in the filtrates (Brian et al., 1952) and is probably the main metabolite responsible for the development of necrotic and chlorotic symptoms (Pound and Stahmann, 1951). This toxin, isolated from lesions or from culture filtrate, causes chlorosis and necrosis when introduced in tomato plants and also damages nonhosts of *Alternaria* such as cabbage, radish, spinach, pea, bean, and others, demonstrating its nonspecificity (Pound and Stahmann, 1951). It was concluded that alternaric acid alters the morphological and physiological characteristics of plasma membranes near plasmodesmata and thereby causes a permeability change, which leads to a leakage of electrolytes (Langsdorf et al., 1991). In cases of less virulent strains or young and therefore resistant leaves, the germinating hyphae of *A. cassiae* and *A. alternata* tend to spread over the intact leaf surface and the only sites of infection are dead cells, suggesting that penetration is preconditioned by the secretion of toxins (van Dyke and Trigano, 1987; Von Ramm, 1962). Those toxins can potentially be used to select for resistant genotypes *in vitro*, either in a purified form or as culture filtrates. The main condition for this is that the plant reaction to the components of the culture filtrate used is similar to the reaction towards *A. solani*.

The objective of this project is to better understand the pathogenic differences between isolates of *A. solani* on potato cultivars. We have previously determined the re-

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sistance phenotypes of nine potato cultivars to five isolates of *Alternaria solani* (Shahbazi et al., 2010). Two potato cultivars, resistant 'Diamond' and susceptible 'Granula', and two pathogen isolates with significantly different pathogenicity phenotypes, named A and N, were selected for further analysis. Here, we monitored the effect of exudates from liquid grown cultures of *A. solani* isolates A and N on cultivars 'Diamond' and 'Granula' using whole seedlings and detached leaflets. We hypothesized that phytotoxins present in the liquid exudates of the two isolates would elicit distinct effects on the host plants. Our results indicate that isolates of *A. solani* exhibiting different degrees of pathogenicity produce phytotoxins with corresponding effects on potato.

Materials and Methods

Cultural conditions. Potato (*Solanum tuberosum*) seeds were planted in plastic pots (30 cm diameter) containing soil and perlite under greenhouse conditions (18-25 °C) at Tehran University. Potato seed consisted of small whole tubers weighing approximately 50 g each. One seed piece was planted per pot. Tubers were treated with thiabendazole (tekto WP60%, Golsam Gorgan Company, Tehran) before planting. Plants were irrigated once per day or as needed.

***Alternaria solani* isolation and spore production.** Potato leaves infected with *Alternaria solani* were collected in 2008 from commercial potato fields in Iran. Two isolates of *A. solani* were recovered from sections of potato leaves with early-blight lesions. An isolate, named "A", was previously characterized as being highly aggressive on potato and a second isolate, named "N", was less aggressive (Shahbazi et al., 2010). The isolates were maintained on PDA at 20 °C for 7 days. To induce sporulation, cultures were grown for 6 days at 23-25 °C on PDA under near-ultraviolet light (310-400 nm) with 16 h/day light. Conidia were collected by washing plates with sterile water and the resulting spore suspension was adjusted to a concentration of 10^6 spores/mL.

Greenhouse test. Four plants (45 days old) of *Solanum tuberosum* cvs. 'Diamond' (resistant) and 'Granula' (susceptible) were inoculated separately by spraying with each isolate of *A. solani* at a concentration of 10^6 spores/mL or a sterile water control (Bokshi et al., 2003; Nadia et al., 2007). The plants were covered with clear plastic bags for 24 h to increase humidity and accelerate infection and then grown under normal conditions in the greenhouse at 26 °C (Bokshi et al., 2003; Pelletier and Fry, 1989). The intensity of infection was recorded using the scale described in Table 1 (Rodríguez et al., 2007).

Table 1. Scale for evaluation of the foliar damage caused by *Alternaria solani*

Rating	Description of Symptoms
10	Spots on lower leaves
20	Spots on most of the lower leaves
30	Spots on all lower and some of the middle leaves
40	Clearly developed blight lesions in lower leaves
50	Blight lesions in lower leaves spread to some middle leaves
60	Blight lesions developed in all inferior and most of the middle leaves
70	Blight lesions developed in all lower and middle leaves
80	Blight lesions developed in all lower and middle leaves and spread to upper leaves
100	Total blight (death of the plant)

Culture filtrate. Small disks (2 mm diameter) from 1-week old PDA cultures were placed in 25 ml of sterile liquid medium containing 1.0 g of KH_2SO_4, 0.5 g of $MgSO_4$, 6.0 g of casein hydrolysate, 100 g of sucrose, 1 mg of $FeSO_4$, 0.15 mg of $CuSO_4$, 0.10 mg of $ZnSO_4$, and 0.10 mg of Na_2MoO_4 per liter of distilled water. The medium was adjusted to pH 4.9 with 0.1 M HCl and autoclaved before inoculation. The pH remained at 4.9 after autoclaving. The cultures were incubated for 6 weeks at 24 °C in the dark without shaking (Maiero et al., 1991). The contents of the flasks were filtered through the Whatman no. 4 filter paper. Culture filtrates were stored at 4 °C in the dark up to 10 hours until used.

Phytotoxicity tests of culture filtrates on potato leaves *in vitro*. Aqueous dilutions of 2:1, 5:1, 10:1 v/v (culture filtrate: sterilized water) were prepared and autoclaved for 15 min. In earlier studies of *Alternaria* toxic metabolites, autoclaving did not affect phytotoxicity of culture filtrates (Pound and Stahmann, 1951). Control treatments included dilutions of noninoculated growth media with distilled water. A method similar to that described by (Hernandez et al., 1991) was used; however, without removing the roots. Plantlets from each cultivar were inoculated by placing 45 day-old whole *in vitro* plantlets in a 15 × 2 cm test tube containing 5 ml of the culture filtrate dilutions or control treatments containing corresponding dilutions of noninoculated media. Leaflets of 4-week old grown plants were excised and placed in plastic petri dishes on 9-cm discs of filter paper saturated with 3 ml of culture filtrate dilutions or control treatments containing corresponding dilutions of noninoculated media (Gilchrist and Grogan, 1976; Hanneke et al., 1988; Siler and Gilchrist, 1983). The petri dishes were sealed with parafilm. The test tubes and petri dishes were placed in a growth chamber for 72 h at 22 ± 2 °C, with

Table 2. Scale for evaluation of the damage produced by *A. solani* culture filtrate in potato *in vitro* plantlets (in tubes)

Rating	Description of symptoms
0	No visible symptoms
1	Light chlorosis at basal zone of plantlet
2	Advanced chlorosis at basal zone of plantlet
3	Basal zone slightly necrotic and some leaves of the middle zone show light chlorosis
4	Accentuated necrosis at basal zone and advanced chlorosis in the middle zone of plantlet
5	Dead leaves at basal zone, middle zone slightly necrotic and apical zone with light chlorosis
6	Plantlet necrotic up to the middle zone and apical zone with advanced chlorosis
7	Total collapse of plantlet

Fig. 1. Disease severity scores of greenhouse-grown potato cultivars inoculated with *A. solani* isolates (N and A) or water (CO). Error bars show the standard error among four replications.

a photosynthetic photon flow density of 100 lE/m/s and a day length of 16 h. To evaluate the individual response of *in vitro* plantlets to the culture filtrate in tubes, a scale of 0-7 degrees was used (Table 2; Rodríguez et al., 2007). The detached leaflets were rated for the degree of phytotoxicity in petri dishes on a scale of zero to three in which 0 = no symptoms, 1 = slight necrosis, 2 = moderate necrosis and wilting, and 3 = severe necrosis and wilting (Maiero et al., 1991).

Analysis of results. All experiments were carried out using a factorial model of a completely randomized experimental design in four replicates per treatment. In greenhouse evaluations, the factorial model was 2 × 3 (two potato cultivars × three treatments), in culture filtrate tests it was 2 × 3 × 3 (two cultivars × three treatments × three dilutions). Analysis of variance was performed on data from whole plant inoculation experiments using SAS v9. A Kruskal-Wallis analysis of variance was performed on *in vitro* phytotoxicity tests due to the nonparametric data sets. All data are presented as means of at least four replications.

Results

Greenhouse evaluation. Greenhouse infection assays of two potato cultivars with different isolates of *A. solani* revealed significant differences between hosts. *S. tuberosum* cv. 'Diamond' exhibited lower disease severity with an average score (± std. error) of 16 (± 2) among the two *A. solani* isolates (Fig. 1). Cultivar 'Granula' showed higher disease severity with an average score of 51 (± 2). Variance analysis indicated a significant difference between responses of these two cultivars to *A. solani* infection as well as a significant difference from the water-inoculated control plants, which exhibited no visible symptoms. Between two
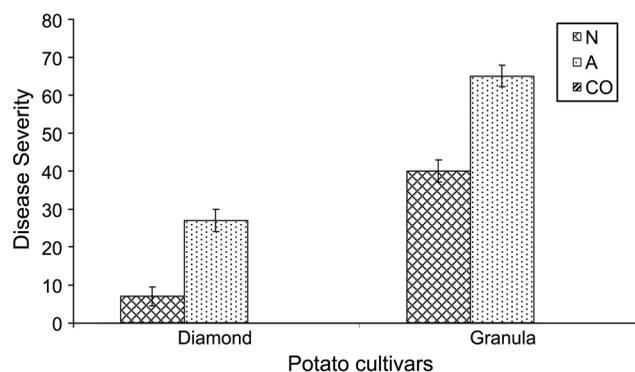
A. solani isolates used in these assays, isolate A exhibited higher aggressiveness with an average host disease severity score of 45 (± 2). Isolate N was less aggressive with an average host score of 24 (± 2).

Culture filtrate in tubes. In order to assess whether EB disease symptoms in potato are caused by exposure to toxins exuded during fungal growth, we exposed whole plantlets to exudates of liquid-grown *A. solani* cultures. Chlorotic and necrotic symptoms developed in *in vitro* plantlets within 72 h after exposure. The severity of symptoms was higher in plantlets exposed to dilutions of the *A. solani* culture filtrate when compared to the control (Fig. 2). The affect on cv. 'Diamond' was lower than cv. 'Granula' with all dilutions (2:1, 5:1, and 10:1) of *A. solani* exudates. Differences in symptoms were observed between
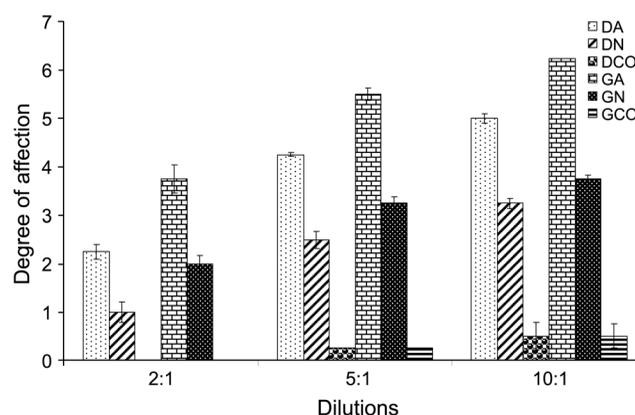
Fig. 2. Phytotoxicity tests of culture filtrates on plantlets *in vitro*. DCO: 'Diamond' in non-inoculated medium culture; GCO: 'Granula' in non-inoculated medium culture; GA: 'Granula' in culture filtrate of *A. solani* isolate A; DA: 'Diamond' in culture filtrate of *A. solani* isolate A; DN: 'Diamond' in culture filtrate of *A. solani* isolate N; GN: 'Granula' in culture filtrate of *A. solani* isolate N. Results are the means of four replications. Error bars denote standard error.
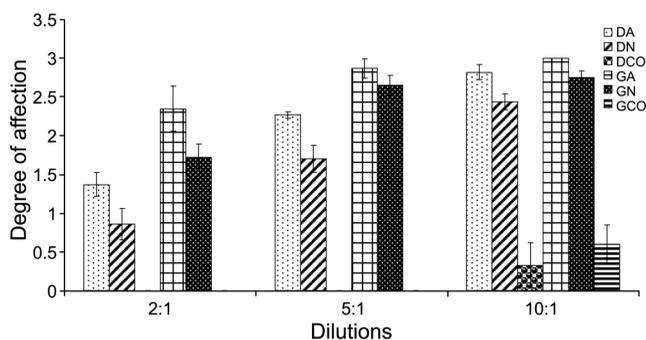
Fig. 3. Phytotoxicity tests of culture filtrates on potato leaves in petri plates. DCO: 'Diamond' in non-inoculated medium culture; GCO: 'Granula' in non-inoculated medium culture; GA: 'Granula' in culture filtrate of *A. solani* isolate A; DA: 'Diamond' in culture filtrate of *A. solani* isolate A; DN: 'Diamond' in culture filtrate of *A. solani* isolate N; GN: 'Granula' in culture filtrate of *A. solani* isolate N. Results are the means of four replications. Error bars denote standard error.

the two cultivars, but were not statistically significant. In both cultivars, symptom expression increased with a higher concentration of fungal culture filtrate and differences between dilutions were significant at P = 0.017.

Differences in host symptom expression were also observed when using isolates of *A. solani* with different aggressiveness phenotypes (Fig. 2). Culture exudate from *A. solani* isolate A, which is more aggressive, elicited more symptoms on both cvs. 'Diamond' and 'Granula' than the less aggressive isolate N and this difference was significant at P < 0.001. The symptoms elicited by the two exudates were also significantly different from those elicited by the control regardless of the dilution used. In an analysis of reactions between isolates, dilution of the exudate of isolate N at a ratio of 5:1 (80%) resulted in approximately the same symptoms as the exudate from isolate A diluted 2:1 (66.7%). Compared to the fungus-free control, both culture filtrates of *A. solani* isolates elicited significantly more symptoms regardless of the dilution.

Culture filtrate in petri dishes. We also assessed the reaction of mature detached leaves to exposure to fungal exudates. In these experiments, the degree of symptoms in leaves of resistant cv. 'Diamond' was significantly less when compared to leaves of cv. 'Granula' (P = 0.010). Differences in symptoms elicited by the two *A. solani* isolates were less pronounced in detached leaves compared to the whole-plant assays although the distinction was still significant at P < 0.001 (Fig. 3). Symptom expression was also higher than in the control-inoculated leaflets regardless of the isolate. Dilution of the fungal exudates also resulted in significant differences in symptoms in both 'Diamond' and 'Granula' leaflets with more symptoms

observed in the most concentrated solution of fungal exudate (P < 0.001).

Discussion

We have previously shown that there are significant differences among resistance phenotypes of potato cultivars 'Diamond' and 'Granula', as well as multiple cultivars with intermediate phenotypes (Shahbazi et al., 2010). These differences are likely not monogenic traits since a gradient of resistance phenotypes were observed when multiple cultivars were tested. We have also observed significant differences among isolates of *A. solani*. Two of these isolates, A and N, produce significantly more and fewer, disease symptoms, respectively. The phenotypic differences in resistance correlate with an significant increase in phenolic compounds (Shahbazi et al., 2010) and expression of β-1,3 glucanase activity (Shahbazi et al., unpublished data), suggesting that these responses have a role in early blight resistance.

Here, we have shown that pathogenesis of *A. solani* in potato is also correlated with its ability to produce toxin metabolites that elicit disease symptoms in the absence of the pathogen. *A. solani* isolates with higher (A) and lower (N) degrees of aggressiveness were significantly separated by disease severity scores as well as the symptoms elicited by their toxins in detached leaflet assays. Rodríguez et al. (2007) showed that culture medium of *A. solani* causes leaf and stem necrosis in *in vitro* plantlets of Solanum sp. that are phenotypically very similar to those caused by infection by spores. This phenomenon has also been observed elsewhere can be used effectively to select for individuals with increased resistance to early blight (Hernandez et al., 1991; Martinez and Sinclair, 1994; Veitía et al., 2001). Our results support the conclusion that different cultivars of potato contain variable reactions to *A. solani* toxins. Additionally, we have shown that different isolates of *A. solani* can produce toxins that produce more or fewer symptoms on the host, suggesting variability in the quantity or effectiveness of the toxins that are produced.

Two *A. solani* isolates with significantly different degrees of aggressiveness were identified using greenhouse infection assays. The A isolate induced more disease and N induced less disease, regardless of the host cultivar used. Although genotypic differences have not been established for these isolates, the presence of multiple strains of *A. solani* in potato growing regions is not uncommon (Lourenço et al., 2009; van der Waals et al., 2004). Isolates from the same host, such as potato, have been found to be more closely related to one another than those collected from another host, such as tomato (Weir et al., 1998). Therefore, among isolates collected from the same host, any genetic variability

that translates to differences in aggressiveness is of great interest and warrants further study.

The two cultivars 'Diamond' and 'Granula' exhibited different levels of resistance to the two isolates using a detached leaflet assay. Together, differences in interaction between resistant 'Diamond' and susceptible 'Granula' with *A. solani* after 72 hours, suggest that their responses are closely associated with defense responses and that the differences in phenotypes are likely due to variability in sensitivity to metabolites produced during growth. The generation of phytotoxins has been demonstrated in several plant pathogens including individuals from the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, and *Verticillium* (Svabova and Lebeda, 2005). To accelerate disease resistance evaluation, *in vitro* selection of toxin sensitivity has been successfully applied in several crops such as banana, carnation, grapevine, strawberry and wheat (Svabova and Lebeda, 2005). Here we have shown that an increased sensitivity to toxins produced by *A. solani* correlates with increased susceptibility to the pathogen. Furthermore, this sensitivity could be assayed using seedlings as young as 45 days old. Further work is required to determine whether the correlation between toxin sensitivity and resistance can be expanded to other potato cultivars or wild species of potato. We expect that the use of seedlings for *in vitro* evaluation of *A. solani* toxin sensitivity could provide a useful method for early generation selection of individuals in families segregating for resistance to early blight.

Acknowledgements

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